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Iodinated α-tocopherol nano-emulsions as non-toxic contrast agents for preclinical X-ray imaging

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ABSTRACT

Micro-computed tomography (micro-CT) is an emerging imaging modality, due to the low cost of the imagers as well as their efficiency in establishing high-resolution (1–100 μm) three-dimensional images of small laboratory animals and facilitating rapid, structural and functional *in vivo* visualization. However use of a contrast agent is absolutely necessary when imaging soft tissues. The main limitation of micro-CT is the low efficiency and toxicity of the commercially available blood pool contrast agents. This study proposes new, efficient and non-toxic contrast agents for micro-CT imaging. This formulation consists of iodinated vitamin E (α-tocopheryl 2,3,5-triiodobenzoate) as an oily phase, formulated as liquid nano-emulsion droplets (by low-energy nano-emulsification), surrounded by a hairy PEG layer to confer stealth properties. The originality and strength of these new contrast agents lie not only in their outstanding contrasting properties, biocompatibility and low toxicity, but also in the simplicity of their fabrication: one-step synthesis of highly iodinated oil (iodine constitutes 41.7% of the oil molecule weight) and its spontaneous emulsification. After i.v. administration in mice (8.5% of blood volume), the product shows stealth properties towards the immune system and thus acts as an efficient blood pool contrast agent ($t_{1/2}$ = 9.0 h), exhibiting blood clearance following mono-exponential decay. A gradual accumulation predominantly due to hepatocyte uptake is observed and measured in the liver, establishing a strong hepatic contrast, persistent for more than four months. To summarize, in the current range of available or developed contrast agents for preclinical X-ray imaging, this agent appears to be one of the most efficient.

1. Introduction

In recent years, various medical imaging technologies have been specifically developed for preclinical research, notably in the field of oncology. In accordance with ethical guidelines on animal experimentation, these new research tools help reduce the number of animals used for experimental protocols. Generally speaking, all imaging modalities have specific limitations that constrain their scale of use and development. If the prohibitive price of imagers or the cost and toxicity of the contrast agents are limiting factors, the supply, storage and management of radioactive animals and wastes (with nuclear imaging, PET, SPECT) are no less problematic. Optical imaging is an emerging modality, very promising due to the relatively lost cost of imagers, but with drawbacks such as very low signal penetration in the animal body, a low spatial resolution and no signal for non-labeled tissues (thus making it impossible to obtain anatomic images). Multimodal imaging is therefore an increasingly popular solution, making the most of the complementarities between various modalities. The second most efficient

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and cost-effective modality after optical imaging is computed tomography (X-ray scanner). However, the main limitation of X-ray imaging for preclinical research is the high cost, low efficiency and non-negligible toxicity of the contrast agents. The present study focuses on the development of X-ray contrast agents aimed at overcoming these limitations.

Micro-computed tomography (micro-CT) is an imaging modality which enables rapid three-dimensional, radiographic, structural and functional visualization in small laboratory animals [1–4]. Compared to clinical CT, the resolution of micro-CT is significantly better, from 1 to 100 μm [5–7], allowing, for instance, the clear detection of metastasis sizing around 300 μm [8–10]. However, the acquisition time of a micro-CT apparatus is slow, around 10–20 min for a full high resolution autoradiogram, whereas the same result can be achieved within seconds with a clinical CT scan on humans [11,12]. X-ray contrast agents developed for humans and used in clinical CT scans are hydrophilic iodinated molecules with a low molecular weight and therefore undergo very fast blood elimination via the kidneys, mainly due to glomerular filtration. For this reason, they are not adapted to preclinical research with micro-CT. Moreover, clinical contrast agents administered at high doses or with repeated injections (to allow micro-CT imaging) have severe drawbacks, leading to acute kidney toxicity, a tendency to extravasate, and allergic reactions [13–15]: thus they should clearly be avoided for micro-CT imaging.

Over the past decades, new micro-CT compatible contrast agents have been developed for X-ray imaging [16–25]. Their particularity is a long residence time in the bloodstream and/or an ability to target specific organs or lesions. In order to prevent renal clearance, these contrast agents have generally been formulated as nanoparticulate systems. The optimized properties of such contrast agents can easily be summarized in five points: (*i*) in order to avoid being eliminated by the kidneys, contrast agents need to be formulated in the form of nanoparticulate systems (liposomes, nano-emulsions, dendrimers, polymeric nanoparticles etc.) with a minimal size of around 100 nm [11,25–32]. (*ii*) In order to confer them with stealth properties, their surfaces have to be controlled or functionalized by grafting on hydrophilic polymers such as polyethylene glycol (PEG) [33–37]. An extended circulation time in the bloodstream is directly linked to the nanometric size range of the contrast agents along with the surface functionalization, preventing rapid opsonization by the reticulo-endothelial system (RES) [34]. (*iii*) Nanocarriers need to contain a great quantity of X-ray contrasting materials (commonly iodine), ideally around 100 mg (or more) of iodine per milliliter of suspension to be administrated [25,28,31]. (*iv*) NP suspensions must be stable for storage and have a high *in vivo* stability, which also affect the stealth properties and residence time in the blood pool. (*v*) In spite of the high loading of contrast agents, the NP suspension must remain non-toxic and neutral to the biological metabolism.

Micro-CT contrast agents are of prime interest in both structural and functional imaging, enabling the detection of lesions through the specific targeting of tissues, *e.g.*, tumors. Targeting tissues, organs or pathologies serve as a new tool to meet the needs of researchers. It can notably provide a better detection of tumors and a follow-up of treatment response allowing the visualization of the tumor growth in time, and thus the *in vivo* efficiency of a therapy. 70% of medical imaging involves cancer research and the design and development of efficient, cost-effective, targeted contrast agents constitute a major research and economic concern. Contrast agents for X-ray imaging modalities are a challenge today: they offer huge potential in terms of advanced diagnosis of tumors and personalized therapies and yet commercial solutions available to date are far from satisfactory.

In this study, we propose an efficient new non-toxic contrast agent for preclinical X-ray imaging. The idea was (*i*) to base the formulation on non-toxic lipophilic molecules naturally present in the body: α-tocopherol (*i.e.* vitamin E), (*ii*) to graft a high ratio of X-ray contrasting material (tri-iodobenzene) on their chemical structure using the simplest chemical reaction, and (*iii*) to formulate nano-emulsions with this iodinated lipid by low-energy nano-emulsification methods with a PEGylated non-ionic surfactant. We chose iodine since it is a compromise between safety, toxicity and cost [28]. This simple process results in the formulation of highly iodinated α-tocopheryl 2,3,5-triiodobenzoate nano-emulsions. When administered intravenously to mice, they show outstanding contrast enhancements, first of the blood compartment and then of the liver tissues through a passive targeting mechanism, and without apparent toxicity. In addition to experiments on the micro-CT imaging and *in vivo* contrasting properties, a complete characterization was performed: *in vitro* biocompatibility studies (hemolysis tests, stability in serum), cytotoxicity studies (MTT), *in vitro* cellular uptake assays, physico-chemical characterization of the nano-emulsions (size distribution, transmission electron microscopy and evaluation of the iodine content), and finally an *in vivo* follow-up of the contrast agent biodistribution.

2. Experimental section

2.1. Materials

2,3,5-Triiodobenzoic acid, α-tocopherol, 4-dimethylaminopyridine, N,N′-dicyclohexylcarbodiimide, dichloromethane, ethyl acetate, cyclohexane and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetra-zolium bromide (MTT) solutions were purchased from Sigma Aldrich, France. Non-ionic surfactant (Cremophor ELP®) from BASF (Ludwigshafen, Germany), was kindly donated by Laserson, Etampes, France. Cremophor ELP® is a parenteral grade nonionic surfactant made by reacting ethylene oxide to castor seed oil at an ethylene oxide to oil molar ratio of 35 [38]. The product mainly consists of a PEG chain (35 ethylene glycol units) grafted onto a molecule of castor oil. Phosphate buffered saline (PBS) and sheep erythrocytes were obtained from Eurobio, France.

2.2. Methods

2.2.1. Synthesis and characterization of α-tocopheryl 2,3,5-triiodobenzoate

The 2,3,5-triiodobenzoic acid (5 g, 0.01 mol), 4-dimethylaminopyridine (0.18 g, 0.0015 mol) and N,N′-dicyclohexylcarbodiimide (2.3 g, 0.011 mol) were sequentially added at room temperature to a solution of DL-α-tocopherol (3.5 g, 0.008 mol) in dicholoromethane (250 mL). The reaction mixture was stirred overnight at room temperature and the dicyclohexylurea and other precipitates were removed by filtration. The organic phase was then washed twice with saturated aqueous $NaHCO_3$, once with saturated NaCl solution and dried with anhydrous Na_2SO_4. The solvent was removed in vacuum and the oil was then purified by the gradient elution method on silica gel using cyclohexane and ethyl acetate as an eluent. Reaction yields were around 80%. The synthesis scheme of α-tocopheryl 2,3,5-triiodobenzoate is reported in Fig. 1. The resulting product was a light, yellowish viscous oil with a high iodine content of around 41.7%.

^1H spectra were obtained with a Bruker Top Spin 3.0400 MHz spectrometer. $CDCl_3$ chemical shifts are expressed in ppm downfield from tetramethylsilane as the internal standard. The purified α-tocopheryl 2,3,5-triiodobenzoate was then characterized by means of NMR and mass analysis: ^1H NMR ($CDCl_3$, δ/ppm): 8.34 (s, 1H, H^6), 8.05 (s, 1H, H^4), 2.63 (t, 2H, H^{14}), 2.15 (s, 6H, H^{31}, H^{32}), 2.10 (s, 3H, H^{30}), 1.83 (m, 2H, H^{15}), 1.59 (m, 3H, H^{20}, H^{24}, H^{28}), 1.28 (s, 3H, H^{33}), 1.27 (m, 18H, all CH_2), 0.89 (d, 9H, $3CH_3$), 0.88 (d, 3H, $1CH_3$). Mass spectrometry was done in positive mode (APCI+) with CAMAG TLC-MS, Agilent Technologies LC/MSD SL. *m/z* 913.5 ($[M + H]^+$).

2.2.2. Preparation of iodinated nano-emulsions

Nano-emulsions of iodinated α-tocopherol were formulated by the spontaneous nano-emulsification method, as described previously [39–41]. In short, pure α-tocopheryl 2,3,5-triiodobenzoate (0.75 g), was firstly mixed with the non-ionic hydrophilic surfactant (0.5 g), and maintained at room temperature. Phosphate buffered saline (PBS), used as an aqueous phase (1.88 g), was then added to the surfactant/oil mixture under gentle magnetic stirring. This optimized formulation was chosen to give a compromise between the nano-emulsion size and monodispersity, and the iodine content of the suspension. As a result of the process optimization described below (in Fig. 2(a)), this compromise led to a droplet diameter of around 85 nm, with the following formulation parameters: surfactant/